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EXPERIMENTAL STUDIES ON INULASE.

ARTHUR L. DEAN.

IN 1888 J. Reynolds Green[1] reported the discovery of an inulin-splitting enzyme in the Jerusalem artichoke, *Helianthus tuberosus*. The tubers were planted and allowed to grow until the stems were about six inches above the ground. At that time the tissues of the tubers were found to have become much altered, being spongy in the middle part, although the outer part was still firm and contained inulin. A glycerine extract of these tubers was prepared and dialysed free from sugar. Such an extract was found to have the power to change inulin to a reducing sugar. Experiments were carried out in test-tubes and in dialysing tubes, with the result that Green was able to establish quite conclusively that the germinating artichoke contained an enzyme able to cause the hydrolysis of inulin. To this enzyme he gave the name "inulase." It is evident from Green's description that the extracts obtained by him were not very active. This he accounts for by supposing the enzyme to occur in very small quantities.

Some time before Green's paper appeared, Bourquelot had observed that *Aspergillus niger* grew well in media in which inulin was the only carbohydrate. Later, in 1893, he[2] published several short papers in which he recorded finding inulase in *Aspergillus niger*. The inulase was associated with several other enzymes, but Bourquelot was convinced that the cleavage of inulin was the function of a distinct ferment. In another paper[3] he compared the enzymes of *Penicillium glaucum* and *Aspergillus niger*, and notes the fact that inulase is present in the first-named fungus.

[1] On the germination of the Jerusalem artichoke (*Helianthus tuberosus*). Annals of Botany **1**: 223. 1888.

[2] Inulase et fermentation alcoholique indirecte de l'inuline. Compt. Rend. **116**: 1143. 1893. Also in Compt. Rend. Soc. Biol. IX. **5**: 481. 1893. Les ferments solubles d l'*Aspergillus niger*. Bull. Soc. Mycol. France **9**: 230. 1893.

[3] Remarques sur les ferments solubles sécrétés par l' Aspergillus et le Penicillium. Compt. Rend. Soc. Biol. IX. **5**: 653. 1893.

He says nothing concerning the inulase of Penicillium beyond the bare fact of its existence.

Chevastelon[4] was able to cause hydrolysis of the "inulin" from the monocotyledons by the agency of the inulase of Aspergillus.

It seemed of interest to investigate more fully the action of the inulase of Aspergillus and Penicillium, and to discover, if possible, whether or not it was identical with the inulase of the artichoke.

CULTURE METHODS.

Pure cultures of *Aspergillus niger* and *Penicillium glaucum* were obtained, and stock cultures kept on the following medium:

MEDIUM A.—Agar-agar, 10^{gm}; NaCl, 2.5^{gm}; peptone (Grübler's), 2.5^{gm}; beef extract, 1^{gm}; inulin (air dry), 5^{gm}; water, 500^{cc}.

The inulin used in making all culture media was prepared from *Dahlia variabilis* by alcoholic precipitation.

The first experiments were carried out upon Penicillium. A culture fluid having the following composition was prepared:

MEDIUM B.—Inulin, 1.5^{gm}; peptone (Grübler's), 0.5^{gm}; NaCl, 0.5^{gm}; beef extract, trace; water to make 150^{cc}.

Test-tubes containing this liquid were sterilized and inoculated with the spores of Penicillium. The fungus grew well in this medium. Each day the culture fluid of one tube was used to test for the presence of sugar (levulose) in solution. During the first three days no sugar reaction was evident, but after that time a reduction could be obtained by boiling the culture fluid with Fehling's solution. The amount of sugar was small at first, but steadily increased, until, after a week's time, the reaction was very marked.

Five days after inoculation the fluid from several cultures was filtered and heated in a water bath with phenyl-hydrazine hydrochloride and sodium acetate. The osazone formed separated while the solution was in the boiling water bath and had the characteristic crystalline form of glucosazone.

In order to establish the fact that the inulin of the medium was the source of the sugar formed, the following culture fluid was prepared which contained no organic compound except inulin:

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[4] Sur l'inuline d'ail, de la jacinthe, d'asphodèle et de la tubereuse. Jour. Pharm. IV. **2**: 1. 1895.

MEDIUM C.—Inulin, 5^{gm}; KNO_3, 0.5^{gm}; $CaCl_2$, 0.25^{gm}; Na_2So_4, 0.25^{gm}; NaH_2PO_4, 0.25^{gm}; $MgSo_4$, 0.2^{gm}; Fe_2Cl_6, trace; water to make 500^{cc}.

About 50^{cc} of this were placed in each of a number of small Erlenmeyer flasks, which, after sterilization, were inoculated with spores of Penicillium. As in the former case, so here, the fungus grew well, and its growth was followed after a few days by the appearance of a reducing sugar in the culture medium. After nine days of growth the culture fluid contained no inulin.

It was found that *Aspergillus niger* grown in the culture fluids described above caused, after a few days of growth, the same appearance of levulose in the solution. After trying a number of media the following was preferred as producing uniformly vigorous growths of the fungi:

MEDIUM D.—Inulin, 15^{gm}; peptone (Grübler's), 7.5^{gm}; KNO_3, 2^{gm} $CaCl_2$, 1^{gm}; Na_2SO_4, 1^{gm}; $MgSO_4$, 1^{gm}; NaH_2PO_4, 1^{gm}; $FeSO_4$, trace water to make 1500^{cc}.

This solution gave about equally good results when 25^{cc} were placed in small (125^{cc}) Erlenmeyer flasks, or when 75^{cc} were placed in larger (500^{cc}) flasks. Since the growth is almost entirely on the surface, a larger percentage of mycelia is obtained from a given quantity of culture medium if the fluid is in a thin layer. The cultures were grown in the dark at a temperature of 25–30° C.

METHOD OF OBTAINING PREPARATIONS OF INULASE.

Cultures of the fungi were grown as described above until about a week old; the mycelia were then filtered from the culture fluid, washed in distilled water, torn apart with needles, and thrown into a mixture of three parts of absolute alcohol and one part of ether. After remaining in the alcohol and ether about five minutes, the material was filtered off on a Büchner funnel and dried. The mass when ground to a powder yielded, in the case of Aspergillus, a black, and in the case of Penicillium, a grayish-green powder.

The above method was suggested by the procedure recommended by Albert[5] as a simple way to obtain the zymase from yeast. It has proved to be a good method for obtaining very active preparations of the invertin of yeast, and was therefore tried as a method for obtaining inulase from Penicillium and Aspergillus.

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[5] Einfacher Versuch zur Veranschaulichung der Zymase. Ber. Deutsch. Chem. Gesell. 33: 3775. 1900.

The Penicillium powder was examined first and was tested as follows. A few milligrams of the powder were ground with sand and water, and the mixture added to two test tubes containing a solution of inulin together with a little toluol. One of these tubes was boiled and both were placed in the incubator. After ten hours a very slight reducing power was observable in the unboiled solution. After forty-four hours equal quantities of the boiled and unboiled fluids were tested with the same quantity of Fehling's solution. The result was striking, there being a copious red precipitate of cuprous oxide in the test of the unboiled digestion, and a clear blue liquid in the other. Some of the liquid from the unboiled digestion was filtered and an osazone made. It was apparently glucosazone.

Experiments showed that this Penicillium powder would not yield a reducing sugar on auto-digestion, that it was without action upon a starch paste which was rapidly digested by saliva, but that it would invert cane sugar. The action of Penicillium powder on inulin, however, was not due to an ordinary invertin, since the invertin of yeast was found to be without action upon inulin.

The Aspergillus powder possessed a rather stronger inulin-splitting power than the Penicillium powder.

A number of attempts were made to obtain inulase from the culture fluids in which Penicillium and Aspergillus had been grown. The fluid itself, both before and after removal of the sugar by dialysis, failed to show any power to hydrolyse inulin. The precipitate thrown down in the culture fluid by alcohol contained no inulase. Attempts were made to make use of the well-known property of enzymes — of being carried down by precipitates of neutral salts formed in their solutions. Precipitates of barium sulphate, calcium sulphate, or calcium oxalate thrown down in portions of the culture fluid had no power to act on inulin. From these facts it is probable that inulase acts inside the fungal hyphae and is not secreted into the surrounding medium. In this respect it resembles the zymase and endotrypsin of yeast, and like them belongs to the class of endo-enzymes.

INFLUENCE OF THE REACTION ON THE ACTIVITY OF INULASE.

It seemed desirable to ascertain whether the inulase found by Green in *Helianthus tuberosus* has the same properties as the inulase of the fungi; and whether or not the inulase of Aspergillus is identical with that of Penicillium. A thorough investigation of all the properties of these enzyme preparations has not been made. The most distinctive property given by Green is that alkalies are harmful to the inulase of artichoke, and that very dilute acids facilitate its action. To test the influence of the reaction of the solution upon the activity of the inulase of Aspergillus and Penicillium the following experiments were undertaken.

I. An inulin solution was made with 1.7569^{gm} of inulin dissolved in 155^{cc} of water containing a small amount of toluol. Of this solution 75^{cc} were placed in each of two flasks. To flask *A* were added 30^{cc} of a suspension of 1^{gm} of Aspergillus powder in 35^{cc} of water; to *B*, 30^{cc} of distilled water. Thirteen test-tubes were filled as follows:

No. 1. 10^{cc} from flask *A*[6] + 10^{cc} of 0.0002 normal H_2SO_4
No. 2. 10^{cc} from flask *A* + 10^{cc} of 0.002 normal H_2SO_4
No. 3. 10^{cc} from flask *A* + 10^{cc} of 0.02 normal H_2SO_4
No. 4. 10^{cc} from flask *A* + 10^{cc} of 0.1 normal H_2SO_4
No. 5. 10^{cc} from flask *A* + 10^{cc} of distilled water
No. 6. 10^{cc} from flask *A* + 10^{cc} of distilled water and boiled
No. 7. 10^{cc} from flask *A* + 10^{cc} of 0.0002 normal KOH
No. 8. 10^{cc} from flask *A* + 10^{cc} of 0.002 normal KOH
No. 9. 10^{cc} from flask *A* + 10^{cc} of 0.02 normal KOH
No. 10. 10^{cc} from flask *B* + 10^{cc} of 0.0002 normal H_2SO_4
No. 11. 10^{cc} from flask *B* + 10^{cc} of 0.002 normal H_2SO_4
No. 12. 10^{cc} from flask *B* + 10^{cc} of 0.02 normal H_2SO_4
No. 13. 10^{cc} from flask *B* + 10^{cc} of 0.1 normal H_2SO_4

Each tube was corked and allowed to stand at 35–37° C. for forty-five hours, at the expiration of which each was boiled and allowed to cool. All were filtered, the first few cubic centimeters of each filtrate being thrown away, and 10^{cc} analyzed for levulose by the ordinary gravimetric copper method. The acid solutions without inulase served as controls to show how much of the sugar formed was due to the enzyme and how much to the acid.

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[6] Before each 10^{cc} was removed, the flask was thoroughly shaken in order that equal quantities of the suspended powder might be taken each time.

The results are given below :

No.	Inulase.	Reaction.	Mgms. levulose in 10^{cc}.	Mgms. levulose due to enzyme.
1	Added	0.0001 N. H_2SO_4	65.2	65.2
2	Added	0.001 N. H_2SO_4	41.6	33.8
3	Added	0.01 N. H_2SO_4	52.3	0
4	Added	0.05 N. H_2SO_4	50.5	0
5	Added	Neutral	53.1	53.1
6	Added	Neutral, boiled	....	0
7	Added	0.0001 N. KOH	25.3	25.3
8	Added	0.001 N. KOH	20.0	20.0
9	Added	0.01 N. KOH	....	0
10	Not added	0.0001 N. H_2SO_4	....	....
11	Not added	0.001 N. H_2SO_4	7.8	....
12	Not added	0.01 N. H_2SO_4	50.4	....
13	Not added	0.05 N. H_2SO_4	49.5	....

II. The sixteen test tubes used in this experiment were filled in a way similar to that in experiment I. Each tube was corked and kept for twenty-one hours at 35–37 ° C. At the expiration of that time the fluid containing acid were neutralized with barium carbonate in substance. All were then boiled, filtered, and made up to 50^{cc}, of which 25^{cc} were analysed in each case. Following are the results :

No.	Inulase.	Reaction.	Mgms. levulose.	Mgms. levulose due to enzyme.
1	Added	0.0001 N. H_2SO_4	35.2	35.2
2	Added	0.0002 N. H_2SO_4	28.9	28.9
3	Added	0.001 N. H_2SO_4	23.2	23.2
4	Added	0.01 N. H_2SO_4	38.8	0
5	Added	0.05 N. H_2SO_4	59.0	0
6	Added	Neutral	27.5	27.5
7	Added	Neutral boiled	....	0
8	Added	0.0001 N. KOH	26.9	26.9
9	Added	0.001 N. KOH	7.0	7.0
10	Added	0.01 N. KOH	....	0
11	Added	0.05 N. KOH	....	0
12	Not added	0.0001 N. H_2SO_4	....	....
13	Not added	0.0002 N. H_2SO_4	....	....
14	Not added	0.001 N. H_2SO_4	....	....
15	Not added	0.01 N. H_2SO_4	39.3	....
16	Not added	0.05 N. H_2SO_4	59.2	....

III. Considerable difference is shown between the two tables in regard to the effect of the presence of 0.0001 normal potassium hydroxide in the solution. Therefore the following experiment was tried. To a solution of inulin Aspergillus powder was added and the fluid shaken. Of this suspension 10^{cc} were measured into each of two test tubes; to one 10^{cc} of 0.0002 normal KOH were added, and to the other 10^{cc} of distilled water. Three drops of thymol solution were added to each. After shaking, both were corked and kept in the incubator for fifty-four hours. At the end of that time the solutions were filtered and analyzed for levulose. Solution containing 0.0001 N. KOH gave 32.0^{mgms}; neutral solution gave 36.6^{mgms}.

IV. Experiment IV was tried with the inulase from *Penicillium glaucum.* The solutions were made up and the tubes filled in a way similar to that in experiments I and II. Each tube, after the addition of five drops of thymol solution, was corked and kept at 38–40° C. for seventy-one hours. At the end of that time the solutions containing acid were neutralized with barium carbonate and all were boiled. After cooling they were filtered through dry filters and each residue washed with 10^{cc} of distilled water added in two portions of 5^{cc} each. The filtrate and wash water were united in each case and 25^{cc} of the solution analyzed for levulose. The results are given in the table below.

No.	Inulase.	Reaction.	Mgms. of levulose in 25^{cc}.	Mgms. of levulose due to enzyme.
1	Added	0.0001 N. H_2SO_4	98.5	98.5
2	Added	0.001 N. H_2SO_4	61.5	31.2
3	Added	0.01 N. H_2SO_4	114.5	....
4	Added	Neutral	71.4	71.4
5	Added	Neutral, boiled	....	....
6	Added	0.0001 N. KOH	64.6	64.6
7	Added	0.001 N. KOH	19.1	19.1
8	Added	0.01 N. KOH	....	....
9	Not added	0.0001 N. H_2SO_4	....	....
10	Not added	0.001 N. H_2SO_4	30.3	....
11	Not added	0.01 N. H_2SO_4	116.0	....

V. In order to ascertain whether the enzyme is destroyed, or only inhibited, by the action of acids and alkalies the following experiment was tried. Four test tubes were filled as follows, the inulin solution used containing Aspergillus powder in suspension:

No. 1. 10^{cc} inulin solution + 10^{cc} of 0.02 N. H_2SO_4
No. 2. 10^{cc} inulin solution + 10^{cc} of 0.1 N. H_2SO_4
No. 3. 10^{cc} inulin solution + 10^{cc} of 0.02 N. KOH
No. 4. 10^{cc} inulin solution + 10^{cc} of 0.1 N. KOH

Each tube was corked and placed in the incubator for twenty-four hours, at the expiration of which the solutions were neutralized; each solution was divided into two parts, one of which was boiled. The resulting eight solutions were kept in the incubator for ten days and then examined for levulose. In no case was more sugar present in the unboiled than in the boiled solution. We may conclude, therefore, that the enzyme had been destroyed in each test.

From an examination of the results of the experiments detailed above, it is evident that the optimum reaction for the inulase of *Aspergillus niger* and of *Penicillium glaucum* is very close to 0.0001 normal acid (sulphuric). This corresponds well with Green's statement concerning the inulase from Helianthus, which, he says, "works most advantageously in a neutral or very faintly acid medium, the best proportion of acid being about 0.001 per cent. of hydrochloric acid." Green also says that "alkalies are deleterious," a fact which agrees with the results of the experiments on inulase from Aspergillus and Penicillium. The reaction of the fluid seems to have the same effect on the enzyme obtained from each of the two fungi.

INFLUENCE OF THE TEMPERATURE ON THE ACTIVITY OF INULASE.

In his recent book on the enzymes Effront[7] states that "the optimum temperature of inulase is found between 50 and 60°." He gives no authority for the statement, neither does he bring forward any experimental evidence. Bourquelot says that inulase survives a temperature of 64°, but makes no statement in regard to its optimum working temperature. Green states that inulase acts better at 40° than at room temperature, but loses its activity at between 70 and 80°. The unusually high temperature given by Effront seemed worthy of investigation, and accordingly the following experiments were undertaken to ascertain the optimum temperature for the inulase of *Aspergillus niger.*

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[7] Enzymes and their application. Translated by Samuel C. Prescott. pp. 249–251. New York. 1902.

I. About 1^{gm} of a very active preparation of Aspergillus powder was extracted for 24 hours in 50^{cc} of water containing a little thymol. The extract was then filtered and added to a solution of about 1^{gm} of inulin and the mixture made up to 150^{cc}. Of this solution 20^{cc} were added to each of seven test-tubes and two drops of toluol added to each tube. All the tubes were corked and kept at the temperatures given below for five hours, at the expiration of which period all were boiled and 15^{cc} from each tube analyzed for levulose with the results given below:

Temperature.	Mgms. of levulose.
10°	37.2
20°	45.4
30°	59.6
40°	78.5
50°	93.1
60°	108.7
70°	38.1

II. One gram of Aspergillus powder was extracted for twenty-four hours in 50^{cc} of water containing a little toluol. The extract was then filtered and added to a solution of 1.7^{gm} inulin in 100^{cc} of water and the mixture then made up to 200^{cc}. Of this solution, 20^{cc} were added to each of eight test-tubes and three drops of toluol added to each tube. Number 8 was then boiled. They were all corked and kept for seven hours at the temperatures given below, at the expiration of which period all were boiled and 15^{cc} from each tube analyzed for levulose with the results given below:

No.	Temperature	Mgms. of levulose
1	20°	13.2
2	30°	18.8
3	40°	21.1
4	50°	29.6
5	60°	26.4
6	65°	11.3
7	70°	10.2
8	50° boiled	8.8

III. One gram of Aspergillus powder was extracted for twenty-four hours in 50^{cc} of water containing a little toluol. The extract was filtered and added to a solution of 1.1^{gms} of inulin in 40^{cc} of water and the resulting mixture made up to 100^{cc}. Of this solution 20^{cc} were added to each of five test tubes and three drops of toluol added to each tube. Number 5 was then boiled. All were corked and kept at the temperatures given below for six hours, at the expiration of which all were boiled and 15^{cc} from each tube analysed for levulose with the results given below.

No.	Temperature	Mgms. of levulose
1	40°	42.2
2	50°	55.5
3	60°	51.8
4	65°	19.9
5	50° boiled	20.9

In the first series of tests the optimum temperature was nearer 60° than 50°, but in the second and third series it was nearer 50°. It is noticeable that in the first series of tests the enzyme was evidently present in much larger quantity than in any of the subsequent digestions. In order to discern whether or not the difference in the effect of the temperature was due to a difference in the concentration of the enzyme the following trial was made:

IV. In flask *A* one gram of Aspergillus powder was extracted in 50^{cc} of water plus toluol. In flask *B* 2.5^{gms} of Aspergillus powder were extracted in the same quantity of water. Both extracts were filtered and added to 75^{cc} of the same inulin solution; toluol was added to each, and each solution made up to 150^{cc}. Two series of six test-tubes each were filled from the two solutions, 25^{cc} being placed in each tube. The tests were kept at the temperatures given below for six hours, then boiled, and 20^{cc} from each was analyzed for levulose.

From the results detailed below it appears that the optimum temperature for inulase lies very near to 55°. There is an indication that in more concentrated solutions the optimum temperature is slightly higher. It is a well-known fact that the presence

SERIES A.—Extract from 1^{gm} Aspergillus powder.

Temperature	Mgms. of levulose
40°	22.5
50°	27.1
55°	29.3
60°	25.7
65°	14.7
55° boiled	11.7

SERIES B.—Extract from 2.5^{gms} Aspergillus powder.

Temperature	Mgms. of levulose
40°	59.4
50°	70.6
55°	74.2
60°	71.9
65°	44.9
55° boiled	33.2

of foreign substances—inorganic salts, proteids, etc.—in the solution in which an enzyme is acting will tend to raise the temperature at which destruction of the enzyme takes place, and in many cases to raise its optimum working temperature.[8] It will be of interest to discover whether or not inulase prepared by a more careful method, by which the bulk of all impurities is eliminated, will show a much lower optimum temperature and range itself with those enzymes which act best at about 40° C.

SUMMARY.

1. In confirmation of the results of Bourquelot, inulase was found in the fungi *Aspergillus niger* and *Penicillium glaucum.*

2. The inulase of these fungi does not pass into the culture medium; it is therefore an endo-enzyme.

3. The preparations of inulase from Aspergillus and Penicillium show similar variations in activity due to changes in the reaction of the medium. The enzyme acts most vigorously in a medium containing 0.0001 normal acid (H_2SO_4); greater

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[8] Cf. CHITTENDEN: On digestive proteolysis. pp. 18, 19. New Haven. 1895.—PUGLIESE: Ueber den Einfluss der Erwärmung auf diastatische Fermente. Archiv Gesamm. Physiol. 69: 115. 1898.

strengths of acids are unfavorable, the enzyme being destroyed by 0.01 normal sulphuric acid. Alkalies hinder the activity of inulase, even 0.0001 normal potassium hydroxide being injurious; 0.01 normal potassium hydroxide destroys the enzyme.

4. The optimum temperature for inulase was found to be about 55° C.

In conclusion, I wish to express my great indebtedness to Professor Lafayette B. Mendel for his able assistance in all parts of this work.

SHEFFIELD LABORATORY OF PHYSIOLOGICAL CHEMISTRY,
YALE UNIVERSITY.